Characterization of the C-Protein from Posterior Latissimus Dorsi Muscle of the Adult Chicken: Heterogeneity within a Single Sarcomere

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ABSTRACT   Specific isoforms of myofibrillar proteins are expressed in different muscles and in various fiber types within a single muscle. We have isolated and characterized monoclonal antibodies against C-proteins from slow tonic (anterior latissimus dorsi, ALD) and fast twich (pectoralis major) muscles of the chicken. Although the antibody against "fast" C-protein (MF-1) did not bind to the "slow" isoform and the antibody to the "slow" C-protein (ALD-66) did not bind to the "fast" isoform, we observed that both antibodies bound C-protein from the posterior latissimus dorsi (PLD) muscle.

Here we demonstrate that in the PLD muscle the binding sites of these two antibodies reside in two different C-protein isoforms which have different molecular weights and can be separated by hydroxylapatite column chromatography. Since we have shown previously that both these antibodies stain all myofibers and myofibrils derived from PLD muscle, we conclude that all myofibers in this muscle contain both isoforms within all sarcomeres.

C-protein is a myofibrillar protein (9) located in seven stripes along each half A-band (2, 8, 10) of the myofibrils of striated muscle. After purification, it is composed of a single polypeptide chain of ~140 k-daltons which has low α-helical content and an elongated configuration (9). It binds both subfragment-2 (13) and light meromyosin (5) fragments of myosin. C-protein can bind pure actin filaments (6) and its binding to the I-bands in myofibrils is calcium regulated (7). Although it has been shown that it inhibits the actin activated ATPase of myosin (9), and may play a role in the assembly of thick filaments (4), its precise function remains unknown.

Isoforms of C-protein have been described in rabbit skeletal muscle (1, 16) and a cardiac form also has been identified (3, 16).

To assist in the isolation and further characterization of C-protein isoforms, we have generated a series of monoclonal antibodies (McAbs) against C-proteins from chicken skeletal muscles of selected fiber type. One McAb (MF-1), raised against "fast"-type C-protein from the pectoralis major muscle, does not cross react with the "slow" type present in the anterior latissimus dorsi muscle (ALD). Conversely, another McAb against ALD C-protein (ALD-66) does not cross react with the pectoralis muscle C-protein (11). Thus, these two C-protein isoforms exhibit distinctive immunochemical properties.

Of interest to the present study was our observation that both these McAbs reacted with C-protein(s) in the posterior

latissimus dorsi muscle (PLD). All myofibers and myofibrils could be stained with both antibodies (11).

Two explanations were possible for these observations: (a) there is a single C-protein polypeptide chain that contains the binding site for both these antibodies, or (b) there are two separate C-protein polypeptide chains each of which reacts with one McAb. The second explanation would imply an intrasarcomeric heterogeneity of C-protein (11).

In this report we demonstrate that C-protein from the PLD muscle consists of two components which can be isolated by hydroxylapatite (HA) chromatography. These two C-proteins have different molecular weights and bind different monoclonal antibodies. We conclude that there is intrasarcomeric heterogeneity of C-protein in the PLD muscle of the chicken.

MATERIALS AND METHODS

Myosin Preparation: Myosin was prepared from PLD and ALD muscles of adult white Leghorn chickens. Ten chickens were usually necessary for each preparation. We used the same procedure reported previously (11) but with phenylmethylsulfonyl fluoride (0.1 mM) and leupeptin (5 μg/ml) present in the low salt homogenization buffer and during the ultracentrifugation step to minimize the proteolytic degradation of C-protein.

C-protein Preparation: C-protein was obtained from the myosin preparation using DEAE-Sephadex A-50 (9). A 16 × 420-mm column with a 400-ml linear gradient was used for PLD myosin. For ALD myosin, a 15 × 220-mm column with a 200-ml linear gradient was used. The voided protein was pooled and precipitated with ammonium sulfate (11). Samples were further

fractionated in a 15 × 170-mm hydroxylapatite column with a 180-ml linear gradient as described (11).

Solid Phase Radioimmunoassay (RIA): RIA was performed as described (11). Protein concentrations were estimated using an extinction coefficient of 1.09 $mg^{-1}ml^{-1}cm^{-1}$ for C-protein and 0.56 $mg^{-1}ml^{-1}cm^{-1}$ for myosin at 280 nm (9). Undiluted McAb supernatant was used for the antigen dilution curves, and 1 μg of antigen was used for the antibody dilution curves.

Immunoblots: SDS PAGE (7.5% acrylamide) (11), in which the stacking gel was polymerized without a comb, was loaded with 200 μg of crude myosin in sample buffer (50 mM Tris-HCl, 5% β-mercaptoethanol, 2.5% SDS, 15% glycerol pH 6.8) over a 10-cm gel width. Alternatively, different samples were compared by running them in the same gel. After electrophoresis, one vertical strip of the gel was stained with Coomassie Blue, and the remainder used for immunoblots on nitrocellulose paper as described (11, 15). Autoradiograms were printed in reverse contrast.

Antibodies: Monoclonal antibodies MF-1, ALD-66, and ^{125}I-Goat anti-mouse IgG were prepared as described (11).

RESULTS

Crude PLD myosin was first fractionated by DEAE-Sephadex chromatography. C-protein and other contaminants were not retained by the column whereas myosin could be eluted with an ascending gradient of KCl (Fig. 1A). One microgram of protein from selected fractions across the column was assayed by solid phase RIA against MF-1 (the pectoralis specific antibody against C-protein), ALD-66 (the ALD-specific antibody) and MF-20 (a McAb against myosin heavy chain). It was observed that C-protein was restricted to the first OD_{280} peak
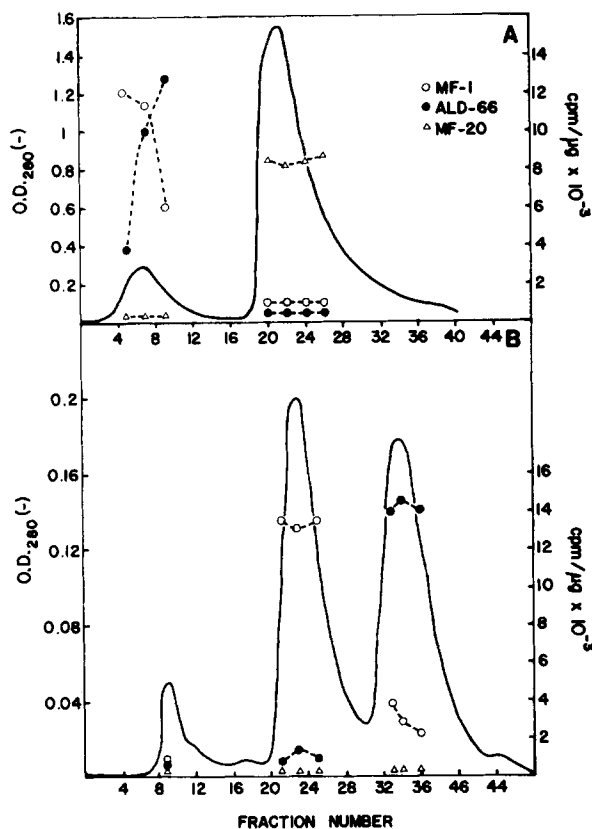

Figure 1 (A) DEAE-Sephadex A-50 chromatography of PLD myosin. 1 μg of total protein from selected fractions was assayed by solid phase RIA with undiluted monoclonal antibodies MF-1 (O); ALD-66 (●), and MF-20 (△), an antibody to myosin heavy chain. Fraction volume: 7.5 ml. (B) Hydroxylapatite (HA) chromatography of PLD C-protein. 1 μg of protein from selected fractions was assayed by solid phase RIA with undiluted antibodies MF-1 (O); ALD-66 (●), and MF-20 (△). Fraction volume: 3 ml.

whereas myosin eluted in the second peak (Fig. 1A). Although both McAbs to C-protein would bind to antigens present in the first peak, we observed that the antigen recognized by MF-1 always eluted slightly ahead of the antigen binding ALD-66 (Fig. 1A).

To better resolve these two antigens, we fractionated C-protein by hydroxylapatite (HA) chromatography. In addition to a small peak that failed to bind either antibody, two major peaks were observed (Fig. 1B). By solid phase RIA with the same three McAb, we could demonstrate that the first major peak contained the antigen binding MF-1 but not ALD-66 whereas the second major peak contained the antigen binding ALD-66 and a small amount of an antigen which reacted with MF-1 (Fig. 1B).

Antigen and antibody dilution assays were performed with the C-protein samples before HA chromatography (Fig. 2A and B) and then compared with the same assays using the central fractions of the first (Fig. 2C and D) and second major protein peaks (Fig. 2E and F). We observed that, although both antibodies would bind equivalently to unfractionated C-protein, almost complete separation of the antigens for these two antibodies was obtained by HA chromatography. The first peak contained only the MF-1 binding antigen while the second peak reacted mainly with ALD-66. We interpret this result to indicate a slight carryover of C-protein from the first into the second C-protein peak, since the binding values for MF-1 decreased in the later eluting fractions of the second peak.

Gel analyses of the samples obtained during these purification steps are shown in Fig. 3. It was possible to resolve two polypeptides in the C-protein region of crude myosin from PLD muscle (Fig. 3A). These two bands were removed from myosin upon DEAE chromatography (Fig. 3B) and were present in almost equal amounts in the crude C-protein preparation (Fig. 3C). The first major peak eluting from the HA column mainly contained the higher molecular weight polypeptide (Fig. 3D), whereas the second peak contained the faster-migrating polypeptide (Fig. 3E).

Varying amounts of a polypeptide of a molecular weight similar to that observed in the second major peak from the HA column was observed in the first peak (Fig. 3D). Immunoblots of the proteins in these two peaks confirmed the specificities observed by RIA and demonstrated that this second polypeptide in the first HA peak reacted exclusively with MF-1. These immunoblots also exhibited McAb binding to smaller fragments of C-protein (see Fig. 5). To support our interpretation that the presence of this lower molecular weight fragment in the first peak was due to partial proteolysis of C-protein during its purification, we performed immunoblots using crude PLD myosin which could be prepared in less than 8 h after tissue removal from the chickens. With these latter preparations, no binding to smaller molecular weight polypeptides was observed with either antibody (Fig. 4).

Further evidence that the two C-protein antigens are of different molecular weight was obtained in these immunoblot experiments. The antigen binding MF-1 migrated more slowly than that binding to ALD-66 (Fig. 4B and C). When both McAbs were mixed, a thicker reactive band was seen in the autoradiograms than if either of the antibodies was tested individually (Fig. 4D).

HA chromatography of ALD C-protein showed only one major peak which bound ALD-66 exclusively (data not presented).
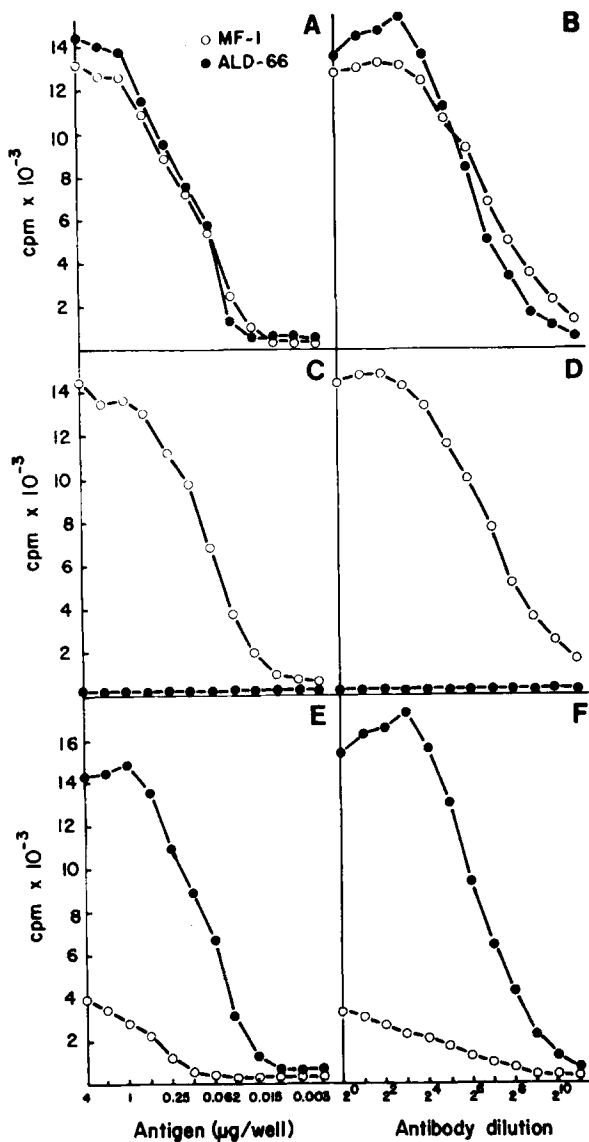
FIGURE 2 Antigen (A, C, and E) and antibody (B, D, and F) dilution curves testing the binding of MF-1 (O) and ALD-66 (●) to crude PLD C-protein (A, B), first peak of the HA column (C, D), and second peak of the HA column (E, F), as determined by solid phase RIA.

individual C-protein polypeptides have been separated by HA chromatography and shown by solid phase RIA to bind only one or the other McAb. Second, the two C-protein polypeptides have been resolved by one-dimensional SDS PAGE, and with immunoblots it was possible to demonstrate the selective binding of the two antibodies to different polypeptides.

The fact that each antibody was specific for only one of the two polypeptides excludes the possibility that the lower molecular weight polypeptide originated from the heavier by proteolysis.

This result, taken together with the fact that both these antibodies bind to all myofibers and myofibrils of the PLD muscle (11), lead to the conclusion that both C-protein polypeptides are contained in all myofibers and incorporated into all myofibrils.

The molar ratio of these two C-proteins after HA chromatography is close to one. If we assume equivalent protein recoveries, the data are compatible with the hypothesis that both polypeptides exist in equal molar concentrations within the intact muscle. This obviously raises the question of their ultrastructural localization in the thick filament. It has been shown that C-protein is localized every 43 nm along the thick filament (2, 8, 10). With fluorescence optics, we did not observe any difference in the immunocytochemical staining pattern of the two antibodies (11). Although purified C-protein is generally considered a monomeric protein (9), the present results are
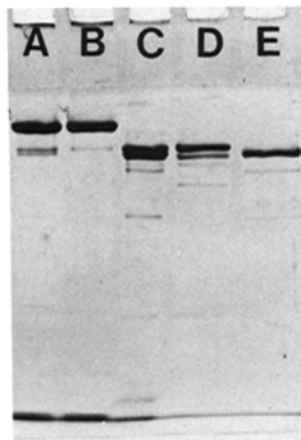

FIGURE 3 SDS PAGE (7.5% acrylamide) of the fractions obtained during the purification of PLD C-protein. Lane A: Crude PLD myosin before DEAE-Sephadex chromatography (2.5 μg). Lane B: PLD myosin after DEAE-Sephadex chromatography (2.3 μg). Lane C: Crude C-protein before HA chromatography (2.1 μg). Lane D: First peak from HA column (1.4 μg). Lane E: Second peak of the HA column (1.2 μg).

It was possible to compare the sizes of C-proteins from different skeletal muscles of the chicken by SDS PAGE (Fig. 5A). The apparent molecular weights were 145 k-daltons for pectoralis C-protein, for the "fast" component of PLD C-protein and for the ALD C-protein. The "slow" component of the PLD C-protein had an apparent molecular weight of 140 k-daltons. Immunoblots of these different C-proteins using the two McAbs confirmed the specificities shown by RIA not only for the main C-protein band but also for the lower molecular weight polypeptides (Fig. 5B and C), suggesting that these polypeptides are partial proteolytic fragments of the original C-protein polypeptide.

DISCUSSION

This study has proven that the binding sites for two different McAbs, which stain all myofibers and myofibrils of the PLD muscle, reside in two different C-protein polypeptides. This has been accomplished by two different methods. First, the
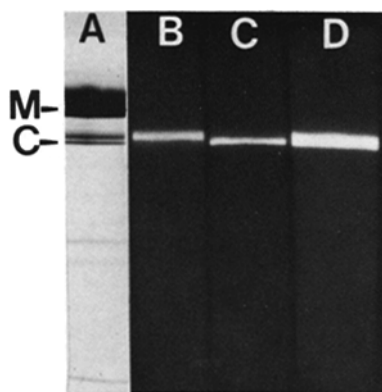

FIGURE 4. Immunoblots of C-protein. Crude PLD myosin was displayed by 7.5% SDS PAGE gel. After electrophoresis, one vertical gel strip was stained with Coomassie Blue (A), the remainder of the gel used for electrophoretic protein transfer to nitrocellulose paper and reacted respectively with MF-1 (B), ALD-66 (C), and a mixture of MF-1 and ALD-66 (D). Autoradiograms were printed in reverse contrast. The arrows indicate myosin heavy chain (M) and C-protein (C).
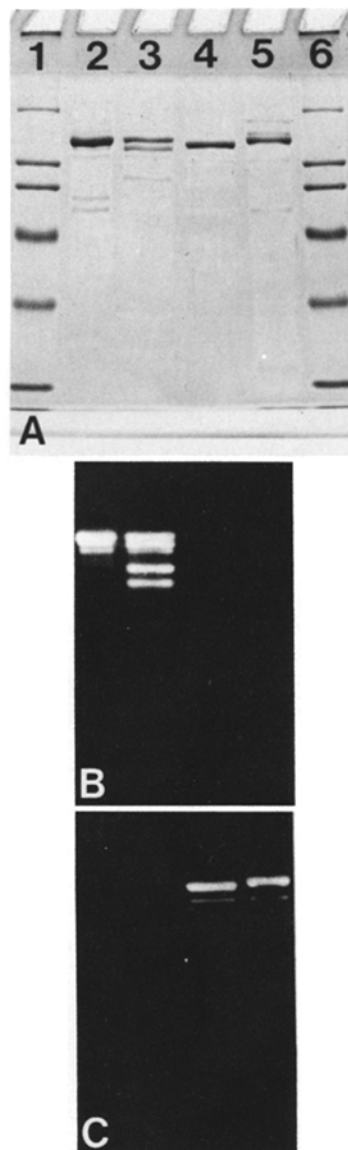
FIGURE 5 SDS PAGE (7.5% acrylamide) of different C-proteins. Lanes 1 and 6 are molecular weight markers of 205, 116, 97, 66, 45, and 29 kdaltons respectively. Lane 2: C-protein from pectoralis major (0.8 μg). Lane 3: "Fast" component of PLD C-protein (0.75 μg). Lane 4: "Slow" component of PLD C-protein (0.75 μg). Lane 5: C-protein from ALD muscle (0.75 μg). A illustrates the Coomassie Blue-stained pattern; B and C contains the autoradiograms of immunoblots of the same C-protein samples reacted with MF-1 and ALD-66, respectively.

nological similarities since both bind MF-1 yet do not bind ALD-66. The ALD C-protein and the "slow" component of the PLD muscle also have immunological similarities since both bind ALD-66 but do not bind MF-1. In this latter case, we could detect a small difference in the molecular weights of these two C-proteins.

In the rabbit, fractionation of C-protein by HA showed the presence of a second polypeptide of similar molecular weight which was termed X-protein (14). The chemical similarities between C- and X-proteins are unknown. On the basis of the present results, however, one must entertain the possibility that C- and X-proteins are isoformic variants.

Precise evaluation of these immunochemical and electrophoretic experiments must await sequence and crystallographic data. The fact that all these polypeptides copurify with myosin, exhibit similar molecular weights, and seem to be localized in the same region of the A-band, suggests that all of them belong to a family of related proteins.

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compatible with a homodimer or heterodimer configuration every 43 nm. The presence of both homo and heterodimers of myosin light chains in the same thick filament have been demonstrated (12). Studies with the electron microscope will be necessary to establish whether these two C-proteins are present at coincident 43-nm repeats.

The exact chemical differences between these two PLD C-proteins are still uncertain. In addition to their immunological differences, they exhibit small molecular weight variations and different chromatographic properties with both DEAE-Sephadex and HA. Only one type of C-protein could be identified in each of the pectoralis major and ALD muscles. The pectoralis type and the "fast" component of the PLD have immu-